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### Salivary gland stem cells

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# Chapter

# 3

## **Salisphere derived c-Kit<sup>+</sup> cells rescue tissue homeostasis in irradiated salivary glands**

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## ABSTRACT

During radiotherapy salivary glands of head and neck cancer patients are unavoidably co-irradiated, potentially resulting in life-long impairment. Recently we showed that transplantation of salisphere-derived c-Kit expressing cells can functionally regenerate irradiated salivary glands. This study aims to select a more potent subpopulation of c-Kit<sup>+</sup> cells, co-expressing stem cell markers and to investigate whether long-term tissue homeostasis is restored after stem cell transplantation.

Salisphere derived c-Kit<sup>+</sup> cells that co-expressed CD24 and/or CD49f markers were intra-glandularly injected into 15Gy irradiated submandibular glands of mice. Particularly, c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> cells transplanted mice improved saliva production ( $54.59 \pm 11.1\%$ ) versus the irradiated control group ( $21.5 \pm 8.7\%$ ). Increase in expression of cells with differentiated duct cell markers like, cytokeratins (CK8, 18, 7 and 14) indicated functional recovery of this compartment. Moreover, ductal stem cell marker expression like c-Kit, CD133, CD24 and CD49f reappeared after transplantation indicating long-term functional maintenance potential of the gland. Furthermore, a normalization of vascularization as indicated by CD31 expression and reduction of fibrosis was observed, indicative of normalization of the microenvironment.

Our results show that stem cell transplantation not only rescue hyposalivation, but also restores tissue homeostasis of the irradiated gland, necessary for long-term maintenance of adult tissue.

## 1. INTRODUCTION

It is estimated that yearly about 500,000 new patients are diagnosed with head and neck cancer worldwide<sup>1</sup> of which many undergo radiotherapy. While the patient's 5-year survival rate after radiotherapy alone or in combination with surgery and chemotherapy may reach 57%<sup>2</sup>, radiation unavoidably damages normal tissues surrounding the tumor. Salivary glands are sensitive to radiation<sup>3</sup>, as patients rapidly develop hyposalivation during radiotherapy, even with uni-lateral sparing<sup>4</sup> and state of the art Intensity Modulated Radiation Therapy (IMRT)<sup>5,6</sup>. Hyposalivation may subsequently lead to xerostomia or dry mouth syndrome, of which the related problems such as oral discomforts, hampered speech and swallowing, dental caries, and oral infection, severely compromise the patient's quality of life<sup>7</sup>. Clinical management of xerostomia is currently unsatisfactory and therefore there is need of alternative treatments.

Late radiation-induced salivary gland damage is pathologically characterized by a loss of epithelial saliva-producing acinar cells<sup>8</sup>, dilated vasculature<sup>9</sup>, altered neuronal innervation<sup>10</sup>, all or not combined with various grades of fibrosis<sup>11</sup>. Moreover, the depletion of the tissue's stem/progenitor cells hinders spontaneous gland regeneration<sup>8</sup>. Accordingly, a strategy to regenerate damaged glands may be achieved by transplantation of stem/progenitor cells ensuring self-maintenance and continuous replenishment of differentiated cells, such as saliva-producing acini. Epithelial stem/progenitor cells communicate with the surrounding niche, or microenvironment, that consists of vascular, stromal and neuronal cells, to balance stem cell processes and ensure tissue homeostasis. Therefore, long-term preservation of both epithelial stem/progenitor cells and their niches become pivotal in future glandular regenerative strategies.

Recently, our group showed the potential of such a therapy in an irradiated mouse model using salivary gland epithelial stem/progenitor cell transplantation to restore long-term saliva production<sup>12, 13</sup>. It is currently proposed that stem/progenitor cells reside in the ducts of salivary glands<sup>14</sup>. Putative markers that can be used to isolate stem/progenitor cells include CD29 (Itga1), CD24, CD49f (Itga6), CD133 (Prom1), Sca1, CD44, CD34, CD90 (Thy1), CD105, CD9, CD81 and c-Kit (CD117), but only few populations were proven to actively restore mouse irradiated glands<sup>12, 13</sup>. To date, in mice the c-Kit<sup>+</sup> cell population appears to have the highest stem/progenitor-like potential, since recurrent transplantations of as few as 100 to 300 c-Kit<sup>+</sup> cells clearly improved post-irradiation saliva production<sup>12</sup>. Transplanted c-Kit<sup>+</sup> cells formed both acini and saliva-transporting ductal structures, making regenerated glands morphologically appear normal.

Interestingly, c-Kit<sup>+</sup> cells are also present in human salivary glands, and can be isolated from cultured salispheres<sup>15</sup>, highlighting their great potential for upcoming clinical usage.

Despite these promising results, several important questions need to be addressed prior to clinical application. Despite the formation of differentiated cell types are c-Kit<sup>+</sup> cells able to; 1) restore the pool of stem/progenitor cells, 2) prevent damage to the microenvironment 3) increase functional regeneration using co-expression with other cell surface markers? Here we describe that transplantation of enriched c-Kit<sup>+</sup> stem/progenitor cells improves functional restoration. Moreover, we show normalization of the stem/progenitor pool and microenvironment indicative for regained tissue homeostasis.

## 2. MATERIALS AND METHODS

### 2.1. Animals

8–12week old female C57BL/6 mice were purchased from Harlan (The Netherlands). The mice were maintained under conventional conditions and fed *ad libitum* with food pellets (RMH-B, Hope Farms B.V., Woerden, The Netherlands) and water. All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

### 2.2. Culturing of murine salivary glands and flow cytometrical analysis

After extirpation, murine submandibular glands were subjected to mechanical and enzymatic digestion, as described<sup>16</sup>. The resulting single cell suspension was cultured into salispheres. After 3 days they were dissociated to single cells using 0.05% trypsin-EDTA (Gibco, Invitrogen) for FACS analysis. Further, the cells were stained with antibodies specific for stem cell markers like Pacific Blue™ anti-mouse CD117 (c-Kit) (Biolegend 105808, San Diego, CA), CD133 (eBioscience 12-1331-82), PE anti-human/mouse CD49f (Biolegend 313613, San Diego, CA), FITC anti-mouse CD24 (BD Biosciences 101820) at 4°C for 20 minutes, followed by a wash step in PBS/0.2% bovine serum albumin (BSA).

### 2.3. Intra-glandular injection of cultured cells

Cell sorting from dissociated spheres for c-Kit<sup>+</sup>, c-Kit<sup>+</sup>/CD24<sup>+</sup>, c-Kit<sup>+</sup>/CD49f<sup>+</sup> and c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> cells was performed using MoFlo flow cytometer (Dako, Carpinteria,

CA). After sorting the cells were suspended in equal volumes of  $\alpha$ -MEM (Gibco, Invitrogen, Carlsbad, CA) containing 2% of fetal calf serum (Gibco, Invitrogen, Carlsbad, CA). Thirty days post-irradiation, under general anaesthesia using a 28G needle and a Microliter Syringe (Hamilton, Reno, NV), both submandibular glands of irradiated mice were injected with a total 10  $\mu$ l of cell suspension with 400 c-Kit<sup>+</sup> cells, 1000 c-Kit<sup>+</sup>/CD24<sup>+</sup> or 400 c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> cells.

## 2.4. Irradiation and saliva collection from mouse salivary glands

Salivary glands were locally irradiated with a single dose of 15 Gy (Precision X-ray Inc. - X-rad 320, 200kV, 20mA, 1.8 Gy/minute) that is known to induce hyposalivation without compromising the general health of the animals. At 120 days post-irradiation saliva flow rate was determined. Animals were placed in a restraining device<sup>17</sup> after pilocarpine injection (2.5 mg/kg, s.c.). Quantity of saliva collected for 15 minutes was determined gravimetrically, assuming a density of 1 g/mL for saliva. Percentage flow rate of saliva of an animal is calculated by denoting the pre-irradiation saliva as 100%.

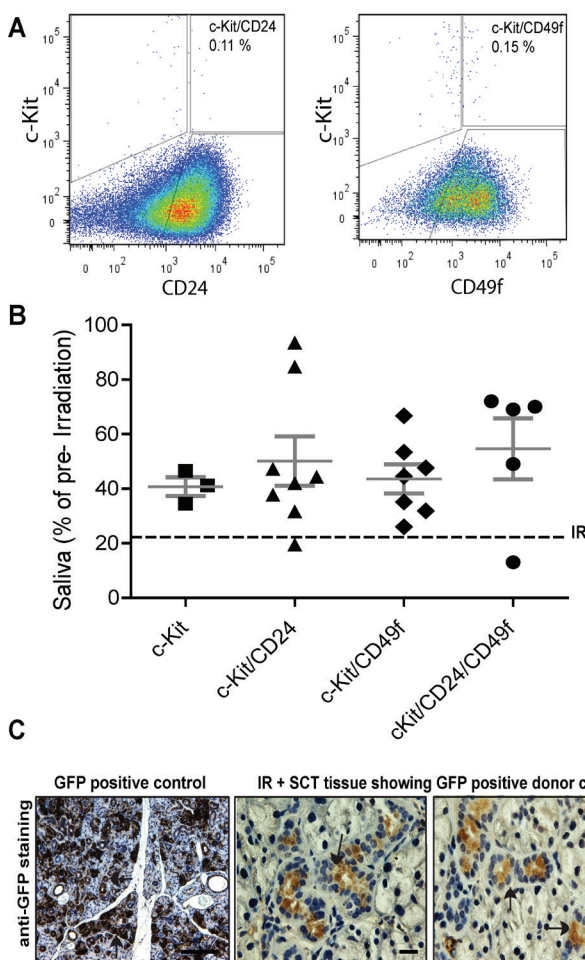
## 2.5. Immunohistochemical analysis of salivary gland

Dissected mouse submandibular glands were immediately embedded in cryopreservation medium, frozen in liquid nitrogen and sliced into 5-25 $\mu$ m sections for cryosectioning. For paraffin embedding, glands were fixed in 4% buffered formaldehyde, following dehydration were embedded in paraffin and sliced into 5  $\mu$ m sections. The sections were dewaxed and labelled for the following markers: c-Kit (1:50, R&D Systems; MAB1356), CD133 (1:50, Abcam; ab19898), CD24 (1:100, Santa Cruz Biotechnology; SC7034), CD49f (1:50, Santa Cruz Biotechnology; SC6596), CD31 (1:100, BD550274), CK8 (DSHB, IA, TROMA-1), CK18 (1:600, Abcam, ab668), CK7 (1:10) (Monosan, MON3007), CK14 (1:20Abcam, ab7800) and Anti-GFP (1:100, Chemicon, MAB3580). Secondary biotin carrying antibodies (Dako, Carpinteria, CA), an avidin-biotin-horse radish peroxidase complex (ELITE ABC Kit, Vector Laboratories, Burlingame, CA) and the diaminobenzidine (DAB) chromogen were added for visualisation under bright field microscopy. Nuclear counterstaining was performed with haematoxylin. Control sections without primary antibodies did not show positive immunostaining. To visualise interstitial fibrosis Masson Trichome staining was performed on paraffin sections.

### 3. RESULTS

#### 3.1. Selection of stem/progenitor cells within c-Kit<sup>+</sup> population

First, the presence of a sub-population of stem cells within salisphere derived c-Kit<sup>+</sup> population was investigated by selecting for co-expression with other stem cells markers.



**Figure.1:** Selection of stem/progenitor cells within c-Kit<sup>+</sup> population. (A) Flow cytometric analysis of salisphere-derived cells showing co-expression of c-Kit cells with CD24 and CD49f. (B) Saliva flow as % of pre-irradiation (100%) in relation to the transplanted sub-populations of c-Kit<sup>+</sup>/CD24<sup>+</sup>, c-Kit<sup>+</sup>/CD49f<sup>+</sup> and c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup>. Each marker is an individual mouse. The dotted line represents average saliva from irradiated (IR), non-transplanted mice. (C) GFP control mouse tissue showing positive (brown) staining with anti-GFP antibody, IR+SCT tissue showing GFP positive duct cells stained with same antibody (Scale bar 50 μm).

Flow cytometric analysis of salisphere derived cells showed that  $0.35\% \pm 0.88$  cells co-express c-Kit<sup>+</sup>/CD24<sup>+</sup> and  $0.14\% \pm 0.09$  are c-Kit<sup>+</sup>/CD49f<sup>+</sup> (Fig.1A) whereas no overlap was

observed with CD133 cells (data not shown). Interestingly, a subpopulation of  $0.03\% \pm 0.02$  c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> triple positive cells could be observed.

Next, the *in vivo* regeneration potential of these cells to ameliorate radiation-induced hyposalivation was evaluated after transplantation into 15 Gy irradiated murine submandibular glands. 90 days later animals transplanted with 400 c-Kit<sup>+</sup> cells showed a significantly improved saliva flow to an average of  $40.7\% \pm 3.47$  (% of pre-irradiation,  $p < 0.05$ ) in comparison to non-transplanted irradiated mice ( $21.5\% \pm 8.7$ ), similar to what was observed in our previous studies<sup>12</sup>. Transplantation of 600 c-Kit<sup>+</sup>/CD49f<sup>+</sup> did not further increase salivary flow (Fig.1B). However, injection of 1000 c-Kit<sup>+</sup>/CD24<sup>+</sup>, or 400 c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> cells showed a larger increase in saliva flow rate to  $50.1\% \pm 9.06$  and  $54.59\% \pm 11.1$ , respectively (both  $p < 0.05$ ). Transplantation of a large (90,000 cells) population of c-Kit<sup>+</sup> cells, which comprise the c-kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> population was published to rescue gland function to a lower extent and only temporarily<sup>12</sup>.

To further investigate the effect of transplantation of putative stem cells on morphology of irradiated salivary glands we performed immuno-histochemistry for several parameters. No large difference in general morphology (Fig.S1A) was observed between glands receiving different subpopulations of c-Kit<sup>+</sup> cells. The number of acinar cells clearly increased as previously published. Therefore, we focused on the ductal compartment known to be important for long-term tissue homeostasis potential. From here on all data will be depicted as irradiated with stem cell transplanted (IR+SCT), irradiated, non-transplanted (IR) and non-irradiated (Control) tissues.

Donor derived cells from transgenic GFP mice were used for the transplantation to visualize the cells using anti-GFP staining. Fig.1C shows duct cells of the recipient salivary glands staining positive for GFP indicating formation of donor-derived cells in transplanted tissue, similar to what has been published before<sup>12</sup>. These results show that transplanted cells can regenerate radiation damaged salivary gland and form new ducts that may contribute to regeneration.

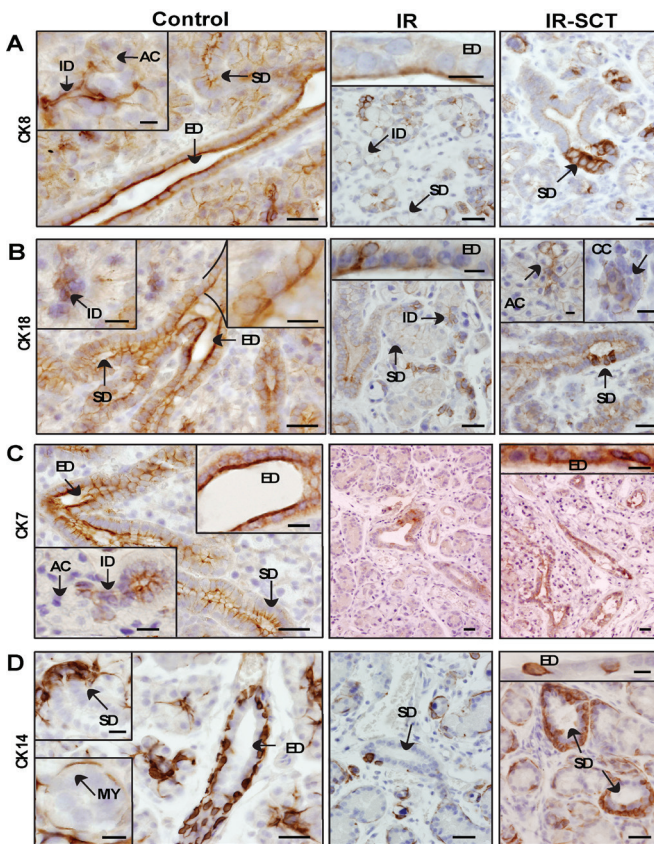
### 3.2. Restoration of tissue homeostasis post-stem cell transplantation

Healthy murine submandibular glands contain saliva-producing acinar cells and the saliva is transported via three different ductal cells namely, smaller intercalated ducts (ID) that collect saliva from acini and striated ducts and bigger excretory ducts (ED) that further release it into oral cavity. Each ductal cell type has distinct expression of cytoskeletal cytokeratins (CK) that regulate cell shape, cell motion and cell division. Expression of CK 8 and CK18 was



observed in acinar and duct cells (Fig.2A & B, control). CK7 is expressed in three different duct cell types, whereas expression of CK 14 is restricted to excretory and striated duct cells and to myoepithelial cells (Fig.2C & 2D, control).

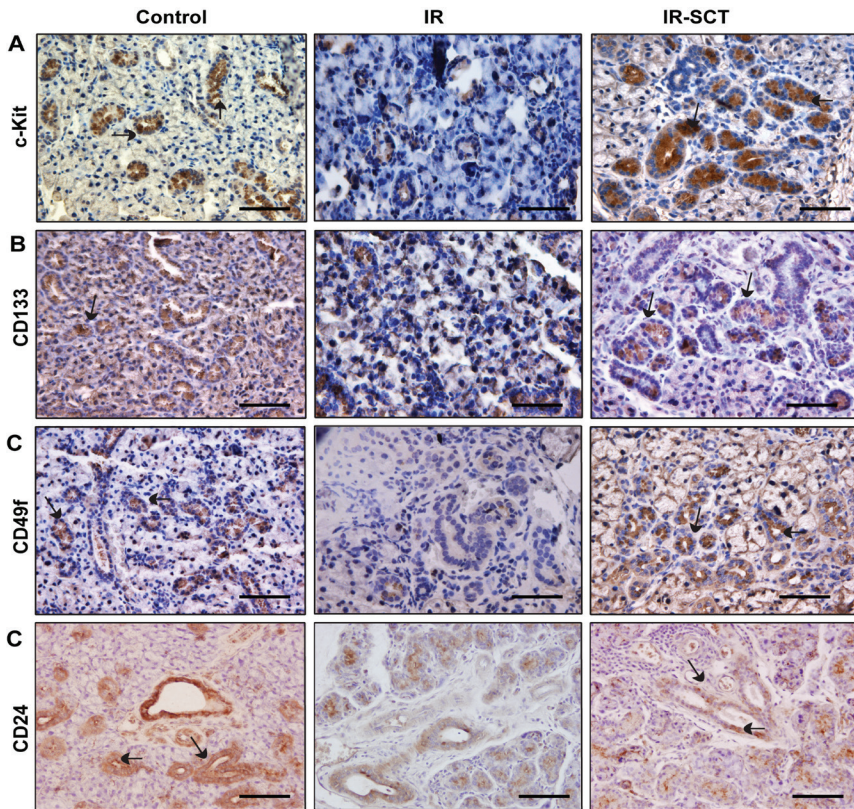
Immuno-histochemical analysis of IR tissue showed a profound decrease in the overall expression of cytokeratins in striated and intercalated duct cells (Fig.2,row IR), whereas the expression of these markers reappeared in the IR+SCT tissue (Fig.2, row, IR+SCT). Interestingly, clusters of newly formed ducts can be observed at various places in the transplanted tissue.



**Figure.2:** Restoration of tissue homeostasis post-stem cell transplantation. Control, IR and IR+SCT tissues stained for duct cell markers CK8 (A), CK18 (B), CK7 (C) and CK14 (D). Arrows indicate positive DAB (brown) staining in excretory (ED), striated (SD) and intercalated duct (ID) cells (Scale bar 50µm).

Proper vascularization is of the up-most importance for salivary gland functioning. Immuno-histochemical staining for endothelial cell marker, CD31 (Fig. S1A, row, Control) in control tissue showed CD31<sup>+</sup> cells lining small blood vessels, whereas in IR tissue the blood vessels are dilated, enlarged (Fig.S1A, row, IR). However post-transplantation in IR+SCT tissue structure of blood vessels seem to be normal (Fig.S1A, row, IR+SCT). In control tissue, neural filament-H staining localizes very limited

number of nerve cells between the epithelial cells (data not shown). In IR tissue due to loss of epithelial tissue, slightly more nerve cells are observed in the spaces, whereas in IR+SCT tissue this is normalized. Masson Trichome staining showed large fibrotic regions (stained blue- Fig. S1C, IR) in the irradiated submandibular glands whereas in IR+SCT tissue little or no fibrosis could be observed similar to that of non-irradiated tissue (Fig.S1B, IR+SCT and Control). From these results it can be concluded that stem cell transplantation regains tissue integrity homeostasis not only by replacing the functional cell loss, but also by preventing the loss of vascularity and preventing fibrosis.



**Figure.3:** Stem/progenitor cells reappear post-transplantation. Control, IR and IR+SCT tissues stained for Stem cell markers c-Kit (A), CD133 (B), CD49f (C) and CD24 (D). Arrows indicate positive DAB (brown) staining in the duct cells (Scale bar 50µm).

### 3.2. Stem/progenitor cells reappear post-transplantation

For long-term homeostasis, however, viable stem/progenitor cells are needed. Therefore, we investigated expression of stem cell markers such as c-Kit, CD133, CD24 and CD49f in the tissues. In control tissue (page 45, Fig.3, Control) bigger excretory duct cells and striated duct cells show the expression of c-Kit, CD133, CD24 and CD49f, which confirms that major ducts of salivary gland contain the tissues' stem/progenitor cells.

Post-irradiation a virtual disappearance of all marker expressing cells was observed (Fig.3 row, IR) whereas in IR+SCT tissue a larger number of ducts that appeared as clusters stained strongly positive for c-Kit, CD49f, CD24 and CD133 (Fig.3, row IR+SCT). Together, these results indicate that stem cells reappear in the entire ductal system, indicative of potential long-term recovery.

## 4. DISCUSSION

Head and neck cancer patients at risk for hyposalivation, as a consequence of therapeutic co-irradiation of salivary glands, may benefit from future stem cell therapies. The aim of this study is to select for enriched stem/progenitor cells within c-Kit cells that enhance functional recovery and to demonstrate whether such stem cell transplantation can recover tissue homeostasis. From previous studies we know that c-Kit cells can contribute to gland regeneration. Since the c-Kit<sup>+</sup> cell population is fairly heterogeneous, functional regeneration by transplanting ~400 cells relies on the inclusion of salivary gland stem cells. Our proposed selection of c-Kit<sup>+</sup> cells co-expressing CD24 and CD49f, showed enhanced functional recovery compared to as few as 400 general c-Kit<sup>+</sup> cells. This concludes that this subpopulation of c-Kit<sup>+</sup> cells is enriched for salivary gland stem/progenitor cells. A consequent step is to demonstrate whether human c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> cells hold similar stem/progenitor-like functions. Since the human salispheres contain c-Kit<sup>+</sup> cells<sup>15</sup>, it is clinically interesting to study if c-Kit<sup>+</sup>/CD24<sup>+</sup>/CD49f<sup>+</sup> cells exist, with any regeneration potential.

Tissue homeostasis is balanced not only by reestablishment of both differentiated and stem/progenitor cells but also by extracellular processes that allow the transport of nutrients and serves reciprocal communication between different compartments. For example, epithelial cells secrete neurotransmitters to stimulate neuronal innervation<sup>10</sup>, which is essential to maintain epithelial progenitor cells<sup>18</sup>.

Maintenance of epithelial stem/progenitor cells<sup>19</sup> and their differentiation<sup>20</sup> also relies

on the presence of endothelial cells, which are recruited by epithelia<sup>21</sup>. Similar processes occur between epithelia and stroma through the exchange of extracellular matrix proteins and growth factors during tissue growth and homeostasis<sup>22</sup>. Radiation alters many of these interaction pathways, whereby changes in the niche may induce an altered environment<sup>11</sup> caused by apoptosis of surrounding cells<sup>23</sup>. Our data suggest that c-Kit<sup>+</sup> cells survive in an irradiated environment and are able to create pools of differentiated acinar cells and putative stem/progenitor cells. Additionally, they improve, at least in part, the morphology of irradiation-compromised niches by enhancing reciprocal communication within niches to establish repair and homeostasis of the tissue. The processes by which these epithelial-niche signaling pathways are restored, however, is still unclear. One possible factor may include neurotransmitters produced by epithelial cells, such as Neurturin, which in *in vitro* settings rescued neuronal innervation after irradiation<sup>10</sup>. The release of cytokines and/or growth factors such as FGF or VEGF may protect blood vessels<sup>9, 24</sup> from further damage as well as restore their ability to migrate and elongate<sup>25</sup>. Similarly, potential candidates for altering fibrosis are the release of MMPs<sup>26</sup> and changes in milieu that reduce reactive oxygen species<sup>27</sup>.

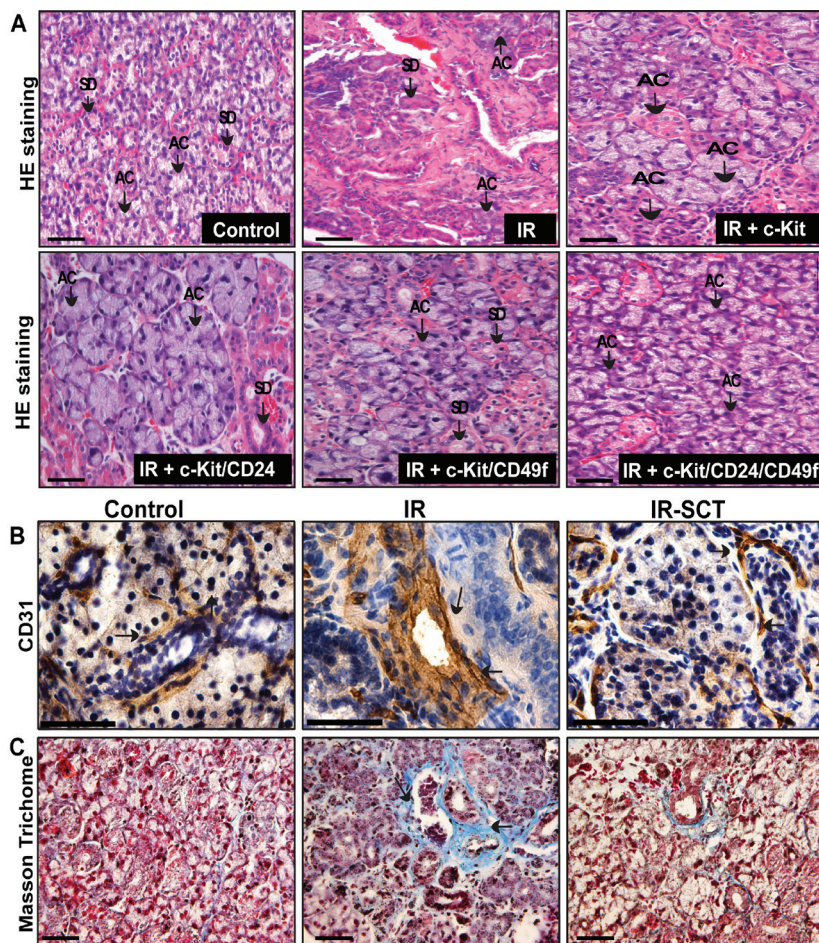
In conclusion, we propose that selecting for stem/progenitor cells within the c-Kit<sup>+</sup> population holds clinical promises for increased regeneration of irradiated salivary glands. Proper stem cell engraftment will also enable the tissue to regain homeostasis by restoring both parenchymal and niche compartments.

## 5. ACKNOWLEDGEMENTS

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## 6. SUPPLEMENTARY INFORMATION



**Figure.S1:** Vasculature and fibrosis are normalized post-transplantation in irradiated tissue. (A) Haematoxylin and Eosin staining showing general morphology of Control, IR and IR+SCT tissues. (B) Control, IR and IR+SCT tissues stained for CD31 and (C) Masson Trichome. Endothelial marker, CD31 staining shows blood vessels in Control, and dilated, enlarged blood vessels in IR tissue, whereas IR+SCT shows normalized vasculature. Fibrotic tissue stains blue in Masson Trichome staining, in Control no fibrotic tissue can be observed, however IR tissue shows lot of fibrotic tissue (blue), in IR+SCT this is very much reduced (Scale bar 50µm).

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